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| <b>(51) International Patent Classification <sup>6</sup>:</b><br><b>C12N 9/00, 9/02, 9/90, A61K 7/06, 7/09</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 95/01425</b><br><b>(43) International Publication Date:</b> 12 January 1995 (12.01.95)                                                                                                                                                                                                                                                                                                                                                                                                            |
| <b>(21) International Application Number:</b> PCT/DK94/00265<br><b>(22) International Filing Date:</b> 28 June 1994 (28.06.94)<br><b>(30) Priority Data:</b><br>0767/93                      28 June 1993 (28.06.93)                      DK<br><b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).<br><b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only):</b> HJORT, Carsten, M. [DK/DK]; Gåseageren 43, DK-4000 Roskilde (DK). DIDERICHSEN, Børge [DK/DK]; Fuglesangsvej 4, DK-3460 Birkerød (DK). AASLYNG, Dorrit [DK/DK]; Fyrren 8, Svogerslev, DK-4000 Roskilde (DK). HANSEN, Christian, K. [DK/DK]; Øster Farimagsgade 93,4, DK-2100 Copenhagen Ø (DK). ANDERSEN, Jens, Tønne [DK/DK]; Skyttebjerg 85, DK-2850 Naerum (DK).<br><b>(74) Common Representative:</b> NOVO NORDISK A/S; Patent Dept., Novo Allé, DK-2880 Bagsværd (DK). |           | <b>(81) Designated States:</b> AM, AU, BB, BG, BR, BY, CA, CN, CZ, FI, HU, JP, KE, KG, KP, KR, KZ, LK, LV, MD, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| <b>(54) Title:</b> COMPOSITIONS COMPRISING PROTEIN DISULFIDE REDOX AGENTS<br><br><b>(57) Abstract</b><br><br>Novel protein disulfide redox agents, DNA sequences expressing these agents, and methods for their production are disclosed. Furthermore, compositions comprising (i) a protein disulfide redox agent in combination with (ii) at least one redox partner, and optionally (iii) at least one or more other enzymes are demonstrated. The compositions can be used for the treatment or degradation of scleroproteins, especially hair, skin and wool, treatment and cleaning of fabrics, as additives to detergents, thickening and gelation of food and fodder, and pharmaceuticals for the alleviation of eye sufferings.                                                                                                                                                                                             |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |

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## COMPOSITIONS COMPRISING PROTEIN DISULFIDE REDOX AGENTS

FIELD OF THE INVENTION

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The present invention relates to novel protein disulfide redox agents, DNA sequences expressing these agents, and methods for their production.

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The invention furthermore relates to compositions comprising (i) a protein disulfide redox agent in combination with (ii) at least one redox partner, and optionally (iii) at least one or more other enzymes. The compositions can be used for the treatment or degradation of scleroproteins, especially hair, skin and wool, treatment and cleaning of fabrics, as additives to detergents, thickening and gelation of food and fodder, and pharmaceuticals for the alleviation of eye sufferings.

BACKGROUND OF THE INVENTION

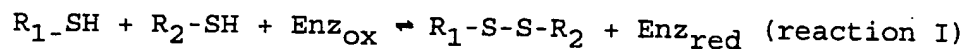
20

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The use of protein disulfide redox agents such as protein disulfide reductases, protein disulfide isomerases, protein disulfide oxidases, protein disulfide oxidoreductase, protein disulfide transhydrogenases, sulfhydryl oxidase, and thioredoxins for various purposes has been known for some time.

Protein disulfide redox agents catalyses the general reaction:

30



35

where  $R_1$  and  $R_2$  represent protein entities which are the same or different, either within the same polypeptide or in two polypeptides,  $\text{Enz}_{\text{ox}}$  is a protein disulfide redox agent in the oxidised state, and  $\text{Enz}_{\text{red}}$  is a protein disulfide redox agent in the reduced state. EC 5.3.4.1 (Enzyme Nomenclature, Academic Press, Inc., 1992) refers to an enzyme capable of catalysing the rearrangement of -S-S- bonds in proteins and EC 1.6.4.4 and

EC 1.8.4.2 is an example of enzymes catalysing the reaction with NAD(P)H and glutathione as a mediator, respectively.

5 This type of activity has in the past been designated as e.g. protein disulfide isomerase, protein disulfide oxidase, protein sulfhydryl oxidase, protein disulfide reductase, sulfhydryl isomerase, disulfide isomerase, protein disulfide transhydrogenase, protein disulfide oxidoreductase and sulfhydryl oxidase.

10 The uses of such enzymes have all been connected with reduction of protein disulfide linkages to free protein sulfhydryl groups and/or the oxidation of protein sulfhydryl groups to protein disulfide linkages, and/or the rearrangement of disulfide linkages in the same or between different polypeptides, and  
15 sometimes with both these processes in sequence.

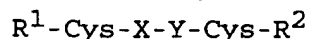
The protein disulfide redox agents of this invention can be divided into four main groups of enzymes, thioredoxin type  
20 (TRX), protein disulfide isomerase type (PDI), disulfide Bond Formation protein type (dsbA) and protein engineered derivatives, chemical modifications and hybrids of TRX and/or PDI and/or dsbA (ENG, sometimes also designated variants or muteins of TRX, PDI or dsbA).

25 TRX is a 12-kDa protein having a redox-active disulfide/dithiol and catalysing thiol-disulfide exchange reactions (Edman et al., Nature 317:267-270, 1985; Holmgren, Annu. Rev. Biochem. 54:237-271, 1985; Holmgren, J. Biol. Chem. 264:13963-13966,  
30 1989).

PDI consists of two subunits, each consisting of two domains which are homologous to TRX.

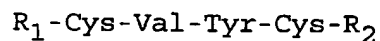
35 DsbA is a 21-kDa protein known to be capable of reducing disulfide bonds of insulin and activity common to disulfide oxidoreductases (Bardwell et al., Cell, Vol. 67, 581-589, 1991).

TRX, DsbA and the two domains in the subunits of PDI generally comprise a sequence which may be represented as below:

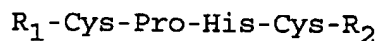


For TRX and PDI are  $R^1$  and  $R^2$  each different amino acid sequences, X generally is Gly, and Y generally is Pro or His, respectively.

TRX from the T<sub>4</sub>-bacteriophage has the sequence:



DsbA from *E. coli* has generally the sequence:



In the context of this invention a protein disulfide redox agent may therefore be defined as an enzyme exhibiting the above sequence, but where X and Y can be any amino acid residue, and catalysing reaction I above.

ENG can be prepared by a variety of methods based on standard recombinant DNA technology:

1) by using site-directed or random mutagenesis to modify the genes encoding TRX, dsbA or PDI in order to obtain ENG with one or more amino acid changes, such as replacements, insertions, and/or deletions,

2) by inhibiting or otherwise avoiding dimerisation of the subunits of PDI, thus giving rise to PDI monomers,

3) by producing partial monomers of PDI, dsbA or TRX, in which regions of the NH<sub>2</sub>- or COOH termini of PDI, dsbA or TRX are lacking,

4) by creating hybrids of PDI, dsbA, TRX and/or ENG,

5) by chemically or enzymatically modifying the products of 1)-4), and

6) by a combination of any of 1)-5).

ENG preparation by standard recombination DNA technology for TRX and PDI according to 1) was described by Lundström et al.

(J. Biol. Chem. 267:9047-9052, 1992) and by a combination of 3) and 5) by Pigiet (WO 8906122).

5 PDI, DsbA, TRX and ENG can be obtained by purification from 1) animal or 2) plant tissues, or from 3) microorganisms, or 4) by expression of recombinant DNA encoding plant, animal, human or microbial PDI, dsbA, TRX or ENG in microorganisms or other suitable hosts, followed by purification of PDI, dsbA, TRX or ENG from extracts or supernatants of said microorganisms.

10 Preparation of TRX according to 1) was described by Luthman and Holmgren (Biochem. 121:6628-6633, 1982), according to 2) by Wada and Buchanan (in "Thioredoxins, structure and function" (Gadal, Ed.) Editions du Centre National de la Recherche Scientifique), according to 3) by Porque et al. (J. Biol. Chem. 245:2362-2379, 1970) and by Laurent et al. (J. Biol. Chem. 239:3436-3445), and according to 4) by Krause et al. (J. Biol. Chem. 266:9494-9500). PDI has been prepared according to 1) by Lambert and Freedman (Biochem J. 213:225-234, 1983), according to 3) by Starnes et al. (US 4632905) and by Hammer et al. (US 4894340), and according to 4) by among others Yamauchi et al. (Biochem. Biophys. Res. Commun. 146:1485-1492, 1987). Finally, an ENG was prepared by Lundström et al. (J. Biol. Chem. 267:9047-9052, 1992) according to 4).

25 Disulfide linkages in proteins are formed between cysteine residues and have the general function of stabilising the three dimensional structure of the proteins. They can be formed between cysteine residues of the same or different polypeptides.

30 Disulfide linkages are present in many types of proteins such as enzymes, structural proteins, etc. Enzymes are catalytic proteins such as proteases, amylases, etc., while structural proteins can be scleroproteins such as keratin, etc, protein material in hair, wool, skin, leather, hides, food, fodder, stains, and human tissue contain disulfide linkages. Treatment of some of these materials with PDI and TRX, and a redox partner has been described previously.

35

5 The use of TRX for waving, straightening, removing and softening of human and animal hair was described by Pigiet et al. (EP 183506 and WO 8906122). Pigiet (US 4771036) also describes the use of TRX for prevention and reversal of cataracts. Schreiber  
10 (DE 2141763 and DE 2141764) describes the use of protein disulfide transhydrogenase for changing the form of human hair. Pigiet (EP 225156) describes the use of TRX for refolding denatured proteins. Use of TRX to prevent metal catalysed oxidative damage in biological reactions is described by Pigiet  
15 et al. (EP 237189).

20 Toyoshima et al. (EP 277563 and EP 293793) describe the use of PDI to catalyse renaturation of proteins having reduced disulfide linkages or unnatural oxidised disulfide linkages, in particular in connection with renaturation of recombinantly produced proteins. Brockway (EP 272781), and King and Brockway (EP 276547) describe the use of PDI for reconfiguration of human hair, and for treatment of wool, respectively. Sulfhydryl oxidase for the treatment of Ultra-high temperature sterilized milk is described in US 4894340, US 4632905, US 4081328 and US 4053644. Schreiber (DE 2141763 and DE 2141764) describes the use of protein disulfide transhydrogenase for changing the form of human hair.



ABBREVIATIONSAMINO ACIDS

|    |   |   |     |   |               |
|----|---|---|-----|---|---------------|
|    | A | = | Ala | = | Alanine       |
| 5  | V | = | Val | = | Valine        |
|    | L | = | Leu | = | Leucine       |
|    | I | = | Ile | = | Isoleucine    |
|    | P | = | Pro | = | Proline       |
|    | F | = | Phe | = | Phenylalanine |
| 10 | W | = | Trp | = | Tryptophan    |
|    | M | = | Met | = | Methionine    |
|    | G | = | Gly | = | Glycine       |
|    | S | = | Ser | = | Serine        |
|    | T | = | Thr | = | Threonine     |
| 15 | C | = | Cys | = | Cysteine      |
|    | Y | = | Tyr | = | Tyrosine      |
|    | N | = | Asn | = | Asparagine    |
|    | Q | = | Gln | = | Glutamine     |
|    | D | = | Asp | = | Aspartic Acid |
| 20 | E | = | Glu | = | Glutamic Acid |
|    | K | = | Lys | = | Lysine        |
|    | R | = | Arg | = | Arginine      |
|    | H | = | His | = | Histidine     |

25 NUCLEIC ACID BASES

|    |   |   |          |               |
|----|---|---|----------|---------------|
|    | A | = | Adenine  |               |
|    | G | = | Guanine  |               |
|    | C | = | Cytosine |               |
|    | T | = | Thymine  | (only in DNA) |
| 30 | U | = | Uracil   | (only in RNA) |

In the Tables "deletions" are indicated by "-", e.g. "SI--AKA" indicating that for this protein it appears as if two deletions have occurred compared to the other proteins in the Tables.

MUTATIONS

In describing the various mutants produced or contemplated according to the invention, the following nomenclatures were adapted for ease of reference:

Original amino acid(s) position(s) substituted amino acid(s)

According to this the substitution of Glutamic acid for glycine in position 195 is designated as:

Gly 195 Glu or G195E  
a deletion of glycine in the same position is:

Gly 195 \* or G195\*  
and insertion of an additional amino acid residue such as lysine is:

Gly 195 GlyLys or G195GK

Where a deletion is indicated in the Tables, or present in a protein not indicated in the Tables, an insertion in such a position is indicated as:

\* 36 Asp or \*36D

for insertion of an aspartic acid in position 36

Multiple mutations are separated by pluses, i.e.:

Arg 170 Tyr + Gly 195 Glu or R170Y+G195E  
representing mutations in positions 170 and 195 substituting tyrosine and glutamic acid for arginine and glycine, respectively.

SUMMARY OF THE INVENTION

The present invention relates to novel protein disulfide redox agents, DNA sequences encoding said novel protein disulfide redox agents, DNA constructs comprising said DNA sequences operably linked to sequences capable of directing transcription and translation of said DNA sequence in a suitable host microorganism, and optionally also being operably linked to sequences encoding signals enabling secretion of said protein

disulfide redox agents encoded by said DNA sequence to the extracellular medium, microorganisms containing said DNA constructs, and a process for producing protein disulfide redox agents.

5 The invention also relates to novel compositions of matter with improved properties for a number of applications and the use of said compositions in said applications. Said compositions comprise (i) a protein disulfide redox agent optionally in  
10 combination with (ii) at least one redox partner, and optionally (iii) one or more other enzymes.

#### BRIEF DESCRIPTION OF THE DRAWINGS AND TABLES

15 Figure 1 displays the plasmid map of pCaHj435 made from the E.Coli expression vector pHD 389 (Lopez - Otin et al., J. Biol. Chem., in press) comprising the dsbA gen sequence.

20 Figure 2 displays the plasmid map of pPL1759 (Hansen. C., Thesis, The Technical University of Denmark, 1992).

25 Figure 3 displays the plasmid map of pJA146 made from the pPL1759 plasmid containing the putative mature dsbA encoding region (J.C.A. Bardwell et al., Cell, 67, p. 581-589, 1991).

30 Table 1 shows an alignment of published eukaryotic PDI amino acid sequences: Bovine (*Bos taurus*) (Yamauchi et al., Biochem. Biophys. Res. Commun. 146:1485-1492, 1987), chicken (*Gallus gallus*) (Parkkonen et al., Biochem. J. 256:1005-1011, 1988), human (*Homo sapiens*) (Rapilajaniemi et al. EMBO J. 6:643-649, 1987), mouse (*Mus musculus*) (Gong, et al., Nucleic Acids Res. 16:1203, 1988), rabbit (*Oryctolagus cuniculus*) (Fliegel et al., J. Biol. Chem. 265:15496-15502, 1990), rat (*Rattus norvegicus*) (Edman et al., Nature 317:267-270, 1985), and yeast (*Saccharomyces cerevisiae*) (Tachikawa et al., J. Biochem. 110:306-313).  
35

Table 2 shows an alignment of PDI amino acid sequences: Alfalfa (*Medicago sativa*) (Shorrosh and Dixon, Plant. Mol. Bio. 19:319-

321, 1992), *A. oryzae*, yeast (*Saccharomyces cerevisiae*) (Tachikawa et al., J. Biochem. 110:306-313), bovine (*Bos taurus*) (Yamauchi et al., Biochem. Biophys. Res. Commun. 146:1485-1492, 1987), rat (*Rattus norvegicus*) (Edman et al., Nature 317:267-270, 1985), and mouse (*Mus musculus*) (Gong, et al., Nucleic Acids Res. 16:1203, 1988).

#### DETAILED DESCRIPTION OF THE INVENTION

The novel protein disulfide redox agents of the invention comprises agents of the type ENG indicated above, wherein one or more of the sequences



has been changed to the following definition:

$R^1$  and  $R^2$  each independently are different amino acid sequences identical to or different from the corresponding sequences in their parent molecule, X and Y each independently are any of the 20 other naturally occurring amino acids different from the ones in their parent molecule with the exception of TRX from *E. coli*, wherein His has been substituted for Pro as Y (*E. coli* P34H), and DsbA from *E. coli*.

$R^1$  and  $R^2$  are preferably of a length between 5 and 500 amino acid residues.

X is chosen among Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg and His, and Y is chosen among Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg and His.

In preferred embodiments X is chosen among Glu, Ala, Ser, Asp, Pro, Val and Y is chosen among Arg, Lys, Asn, Gln or Tyr.

In other preferred embodiments X is Gly, Val or Ala, while Y is any of the 20 other naturally occurring amino acids different

from the ones in their parent molecule, preferably Arg, Lys, Asn, Gln, Tyr or His.

5 In other preferred embodiments Y is either His, Pro or Tyr, while X is any of the 20 other naturally occurring amino acids different from the ones in their parent molecule, preferably Glu, Ala, Ser, or Asp.

10 With the exception of TRX from *E. coli*, wherein His has been substituted for Pro as Y (*E. coli* P34H) and dsbA from *E. coli*.

15 It is contemplated that not all sequences (A) in a specific variant or mutein have been changed in the same way, but it is preferred that all sequences (A) in a specific variant or mutein have been changed in the same way.

20 In yet further embodiments it is contemplated that the parent molecule is divided or truncated so as to provide for a smaller number of sequences (A) in each peptide, e.g. one PDI subunit with 2 (A) sequences or a split PDI, dsbA or TRX unit with only one (A) sequence; or wherein  $R^1$  and  $R^2$  different from those of the parent molecule. Naturally  $R^1$  and  $R^2$  may be made both shorter or longer.

25 The novel protein disulfide redox agents of the invention can be obtained through the methods indicated below.

30 As indicated the invention also relates to DNA sequences coding for the variants of the invention.

35 Many methods for introducing mutations into genes are well known in the art. After a brief discussion of cloning protein disulfide redox agent genes, methods for generating mutations in both random sites, and specific sites, within the protein disulfide redox agent gene will be discussed.

Cloning a protein disulfide redox agent gene

The DNA sequence of the DNA construct of the invention may be isolated by well-known methods. Thus, the DNA sequence may, for instance, be isolated by establishing a cDNA or genomic library from an organism expected to harbour the sequence, and screening for positive clones by conventional procedures. Examples of such procedures are hybridization to oligonucleotide probes synthesized on the basis of any of the full amino acid sequences shown in Tables 1 and 2, or a subsequence thereof in accordance with standard techniques (cf. Sambrook et al., 1989), and/or selection for clones expressing a protein disulfide redox agent activity as defined above, and/or selection for clones producing a protein which is reactive with an antibody raised against the protein disulfide redox agent comprising any of the amino acid sequences shown in Tables 1 and 2.

A preferred method of isolating a DNA construct of the invention from a cDNA or genomic library is by use of polymerase chain reaction (PCR) using degenerate oligonucleotide probes prepared on the basis of the amino acid sequence of the parent protein disulfide redox agent of the invention. For instance, the PCR may be carried out using the techniques described in US Patent No. 4,683,202 or by R.K. Saiki et al. (1988).

Alternatively, the DNA sequence of the DNA construct of the invention may be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beaucage and Caruthers (1981), or the method described by Matthes et al. (1984). According to the phosphoramidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA construct may be of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire recombinant DNA molecule, in accordance with standard techniques.

Generation of random mutations in the protein disulfide redox agent gene

Once the protein disulfide redox agent gene has been cloned into a suitable vector, such as a plasmid, several methods can be used to introduce random mutations into the gene.

One method would be to incorporate the cloned protein disulfide redox agent gene, as part of a retrievable vector, into a mutator strain of *Eschericia coli*.

Another method would involve generating a single stranded form of the protein disulfide redox agent gene, and then annealing the fragment of DNA containing the protein disulfide redox agent gene with another DNA fragment such that a portion of the protein disulfide redox agent gene remained single stranded. This discrete, single stranded region could then be exposed to any of a number of mutagenizing agents, including, but not limited to, sodium bisulfite, hydroxylamine, nitrous acid, formic acid, or hydralazine. A specific example of this method for generating random mutations is described by Shortle and Nathans (1978, Proc. Natl. Acad. Sci. U.S.A., 75: 2170-2174). According to the Shortle and Nathans method, the plasmid bearing the protein disulfide redox agent gene would be nicked by a restriction enzyme that cleaves within the gene. This nick would be widened into a gap using the exonuclease action of DNA polymerase I. The resulting single-stranded gap could then be mutagenized using any one of the above mentioned mutagenizing agents.

Generation of site directed mutations in the protein disulfide redox agent gene

Once the protein disulfide redox agent gene has been cloned, and desirable sites for mutation identified, these mutations can be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a preferred method, a single stranded gap of DNA, bridging the protein disulfide redox agent

gene, is created in a vector bearing the protein disulfide redox agent gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in by DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984, Biotechnology 2:646-639). According to Morinaga et al., a fragment within the gene is removed using restriction endonuclease. The vector/gene, now containing a gap, is then denatured and hybridized to a vector/gene which, instead of containing a gap, has been cleaved with another restriction endonuclease at a site outside the area involved in the gap. A single-stranded region of the gene is then available for hybridization with mutated oligonucleotides, the remaining gap is filled in by the Klenow fragment of DNA polymerase I, the insertions are ligated with T4 DNA ligase, and, after one cycle of replication, a double-stranded plasmid bearing the desired mutation is produced. The Morinaga method obviates the additional manipulation of constructing new restriction sites, and therefore facilitates the generation of mutations at multiple sites.

#### Expression of protein disulfide redox agent mutants

According to the invention, a mutated protein disulfide redox agent gene produced by methods described above, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector. An expression vector generally falls under the definition of a cloning vector, since an expression vector usually includes the components of a typical cloning vector, namely, an element that permits autonomous replication of the vector in a microorganism independent of the genome of the microorganism, and one or more phenotypic markers for selection purposes. An expression vector includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the coding sequence of the gene. For ex-



pression under the direction of control sequences, a target gene to be treated according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can support the transcription of the mutant protein disulfide redox agent gene, include but are not limited to the prokaryotic  $\beta$ -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94.

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*  $\alpha$ -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*),

the promoters of the *Bacillus Amyloliquefaciens*  $\alpha$ -amylase (amyQ), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding A. oryzae TAKA amylase, *Rhizomucor miehei* aspartic proteinase, A. niger neutral  $\alpha$ -amylase, A. niger acid stable  $\alpha$ -amylase, A. niger glucoamylase, *Rhizomucor miehei* lipase, A. oryzae alkaline protease, A. oryzae triose phosphate isomerase or A. nidulans acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the protein disulfide redox agent of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1, pHD 389 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B.subtilis* or *B.licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Examples of *Aspergillus* selection markers include *amdS*, *argB*, *niaD* and *sc*, a marker giving rise to hygromycin resistance. Furthermore, the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. The protein disulfide redox agents of the invention comprising a variant of any of the amino acid sequences shown in tables 1 or 2 may furthermore comprise a preregion permitting secretion of

the expressed protein disulfide isomerase into the culture medium. If desirable, this preregion may be native to the protein disulfide isomerase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombinant production of a polypeptide of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal, an avian, an insect, or a plant cell, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, Ba-

*cillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of transforming *Fusarium* species is described by Malardier et al., 1989.

According to the invention expression of the DNA construct comprising the recombinant DNA sequence or expression vector carrying the DNA construct may take place as heterologous expression in a host cell different from the cell from where the recombinant DNA was derived.

According to the invention expression of prokaryote recombinant DNA may take place heterologously in cell compartments.

In preferred embodiment according to the invention the recombinant DNA derived from a cell e.g. of the genus *Escherichia* can be expressed in an other cell e.g. of the genus *Bacillus* or *Streptomyces*.

In another preferred embodiment of the invention the recombinant DNA derived from a cell e.g. of the genus *Aspergillus* can be expressed in a cell e.g. of the genus *Bacillus* or *Streptomyces*.

In a yet further aspect, the present invention relates to a method of producing a recombinant protein disulfide redox agent of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the protein disulfide redox agent and recovering the protein disulfide redox agent from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the protein disulfide redox agent of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

The resulting protein disulfide redox agent may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, if necessary after disruption of the cells, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

It is of course also possible to produce the protein disulfide redox agent of the invention by culturing the filamentous fungal natural host or parent organism of interest and recovering the protein disulfide isomerase from the culture broth in traditional ways.

The present invention also relates to compositions comprising the protein disulfide redox agents of the invention, including also the variant of TRX from *E. coli*, wherein His has been substituted for Pro as Y (*E. coli* P34H).

The compositions may suitably contain 0.01-200 mg of enzyme protein per gram, preferably 0.01-20 mg of enzyme protein per gram, especially 0.01-2 mg of enzyme protein per gram, or

alternatively 0.02-0.2 mg of enzyme protein per gram, or 0.01-0.2 mg of enzyme protein per gram.

5 In another preferred alternative the composition contain 0.01-0.5 mg of enzyme protein per gram or alternatively 0.2-0.5 mg of enzyme protein per gram.

10 The compositions of the invention usually also comprises (ii) a suitable redox partner.

15 The redox partner (ii) is generally an organic or inorganic reductant, and would often be selected from the organic reductants, such as from the group comprising glutathione, L-cysteine, dithiothreitol, 2-mercaptoethanol, thioglycolic acid, L-cysteine ethylester,  $\beta$ -mercaptoethylamine, mercaptosuccinic acid,  $\beta$ -mercaptopropionic acid, dimercapto adipic acid, thiomalic acid, thioglycolamides, glycol thioglycolate, glycerol thioglycolate, thiolactic acid and salts thereof.

20 Among inorganic reductants sulfite and bisulfite compounds will often be preferred.

25 Under this aspect the invention is not meant to comprise compositions comprising only naturally occurring TRX or PDI in combination with a redox partner. All types of ENG are naturally encompassed by the present invention also under this aspect.

30 Furthermore the compositions of the invention may optionally comprise (iii) another enzyme, where said other enzyme preferably is selected among proteases, lipases, amylases, transglutaminases, or another protein disulfide redox agent

35 Under this aspect the invention is meant to comprise compositions comprising all types of protein disulfide redox agents including naturally occurring TRX or PDI either without or in combination with a redox partner. All types of ENG are nat-

urally encompassed by the present invention also under this aspect.

5 The compositions of the invention may contain other ingredients known in the art as e.g. excipients, stabilizers, fillers, detergents, etc.

10 The compositions of the invention may be formulated in any convenient form, e.g. as a powder, paste, liquid or in granular form. The enzyme may be stabilized in a liquid by inclusion of enzyme stabilizers. Usually, the pH of a solution of the composition of the invention will be 5-10 and in some instances 7.0-8.5. Other enzymes such as proteases, cellulases, oxidases, peroxidases, amylases or lipases may be included in the  
15 compositions of the invention, either separately or in a combined additive.

20 The compositions of the invention can be used for the treatment or degradation of scleroproteins, especially hair, skin and wool, treatment and cleaning of fabrics, as additives to detergents, thickening and gelation of food and fodder, strengthening of gluten in bakery or pastry products, and as pharmaceuticals for the alleviation of eye sufferings.

25 The present invention is further illustrated in the following examples which should not, in any manner, be considered to limit the scope of the present invention.

30 MATERIALS AND METHODS

Strain:

*E. coli* WA803 (Maniatis et al., 1982, Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory, New York)

35 *B. Subtilis* DN1885 (P. L. Joergensen et al., Gene, 96, p. 37-41, 1990)

JA146 : *B. subtilis* DN1885 harbouring the pJA146 plasmid

CaHj435 : *E. coli* harbouring the pCaHj435 plasmid

Plasmids:

pCaHj435: Figure 1, plasmid comprising the dsbA gene sequence in *E. Coli* expression vector pHD389.

5 pJA146: Figure 3, plasmid comprising the putative dsbA encoding region (J.C.A. Bardwell et al. Cell, 67, p. 581-589, 1991) in *B. subtilis* expression vector

pPL1759 .

10 pPL1759: *B. subtilis* expression vector (Hansen C., 1992, Thesis, The Technical University of Denmark), figure 2.

pHD389: *E.coli* expression vector, (Lopez - Otin et al., J. Biol. Chem., in press)

Materials:

15 Perkin Elmer- cetus Amplitaq™ Taq polymerase

DNA sequencing kit Sequenase™ (United States Biochemicals)

Super Taq™ DNA polymerase/PCR buffer, HT Biotechnology Ltd.)

Terrific Broth medium (Maniatis et. al (1982) Supra)

Terrific Yeast medium (PCT/DK90/00332)

20 LB agar (Luria-Bertani medium/agar, C.R. Harwood and S.M. Cutting (Ed.) Molecular Biological Methods for Bacillus, 1990, John Wiley & Sons Ltd.)

DEAS Sphadex A-50 column (Pharmacia Fine Chemicals AB)

25 Methods:

Trypsin inhibitor assay (Available from Novo Nordisk A/S)

N-terminal amino acid sequence analysis

30 N-terminal amino acid sequence analysis of recombinant dsbA was carried out following electroblotting using an Applied Biosystems 473A protein sequencer operated according to the manufacturers instructions.

35 EXAMPLES

Example 1



Construction of the dsbA expression plasmid for expression in *E. coli*.

The *E. coli* dsbA gene sequence was collected from the GenBank database (accession number M77746). Based on this sequence a PCR primer containing the restriction enzyme Cla I recognition sequence and 23 bases of the dsbA 5' coding sequence (primer 5513) and a PCR primer containing the restriction enzyme SalI recognition sequence and 23 bases complementary to the dsbA 3' coding sequence (primer 5512) were constructed.

5513      5' CCATCGATGAAAAAGATTTGGCTGGCGCT      3 '  
5512      5' CCGTCGACTTATTTTTTCTCGGACAGATATT      3 '

Total DNA was extracted from *E. coli* strain WA803 using standard procedures.

This DNA was used without further modification as template in a PCR reaction (20 cycles) using the primers 5513 and 5512 and the Perkin Elmer- cetus Amplitaq™ Taq polymerase following the manufacturer's instructions.

A PCR fragment corresponding to the size of the dsbA gene was recovered from an agarose gel and digested with the restriction enzymes ClaI and SalI.

The *E. coli* expression vector pHD 389 was digested with the same enzymes, and the large vector fragment was ligated to the digested PCR fragment. The ligation mixture was used to transform *E. coli* strain WA803. After 24 hours of growth at 30°C using ampicillin selection a transformant was selected and subsequent DNA sequence analysis using the DNA sequencing kit Sequenase™ showed that a sequence identical to the published dsbA gene sequence was integrated between the lambda PR promoter and the fd terminator. This plasmid was termed pCaHj 435, and the *E. coli* strain harbouring the plasmid was termed CaHj 435. A plasmid map of pCaHj 435 is shown in figure 1.

Expression of dsbA in E. coli phage lambda

The dsbA gene is under control of the promoter PR from the E. coli phage lambda. PR is repressed by CI repressor also harboured by the plasmid pCaHj 435. However the CI repressor allele used in this plasmid is temperature sensitive being active at 30°C but inactive at 42°C. Thus the dsbA gene is repressed at 30°C but expressed at 42°C.

In order to express the dsbA gene the strain CaHj 435 was grown in shake flasks containing the medium Terrific Broth at 30°C and 200 rpm until OD600 reached 0.2. Then the shake flasks were transferred to 42°C (200 rpm) for 18 hours.

Recovery and purification of the dsbA gene product.

1 liter of cell suspension was chilled on ice and then the periplasmic fraction of the cells was isolated by osmotic shock:

The cells were isolated by centrifugation (2500 x g, 15 min.) and resuspended in 100 ml 20% (W/V) sucrose buffered with 10 mM Tris/HCl pH 7.0. EDTA were added to a final concentration of 15 mM. The cell suspension was incubated on ice for 15 min. and then the cells were collected by centrifugation (2500 x g, 15 min.). The cells were resuspended in 70 ml of water by vigorous shaking and subsequently incubated on ice (10 min). The suspension was centrifuged (2500 x g, 15 min.) and the supernatant containing the soluble periplasmic fraction was isolated. Tris/HCl pH 7.0 was added to a final concentration of 5 mM.

The dsbA gene product was then purified by DEAE anion exchange chromatography.

A column containing 20 ml of DEAE Sephadex A-50 purchased from Pharmacia Fine Chemicals AB was equilibrated with 10 mM Tris/HCl pH 7.0. The osmotic shock preparation was applied to the column, and then the column was washed with 200 ml 10 mM Tris/HCl pH 7.0. The dsbA gene product was eluted with 50 ml 50

mM NaCl, 10 mM Tris/HCl pH 7.0. SDS polyacrylamide gel electrophoresis showed that more than 90% of the protein isolated corresponded to the size of the dsbA gene.

5 Using the trypsin inhibitor assay it was shown that the purified protein has disulphide isomerase activity.

#### Example 2

10

#### Construction of the dsbA expression plasmid for expression in *Bacillus*.

The *E. coli* dsbA gene sequence was collected from the GenBank database (accession number M77746). Based on the dsbA sequence from GenBank and pCaHj435 (the dsbA expression plasmid in *E. coli* (WA803)) a PCR primer containing the restriction enzyme NsiI recognition sequence and 27 bases of the dsbA 5' sequence encoding the putative N-terminal (J.C.A. Bardwell et al. Cell, 67, p. 581-589, 1991) of the mature DsbA protein (primer 5965) and a primer containing a restriction enzyme EcoRI recognition sequence and 20 bases complementary to the dsbA 3' sequence of pCaHj435 (primer 5966) was made:

25 5965 5'-CCTCATTATGCATCAGCGGCGGCGCAGTATGAAGATGGTAAACAG-3' 5966  
5'-GCGAATTCGTCGACTTATTTTTTCTCGG-3'

30 A reisolated colony of WA803/pCaHj435 grown 18 hours at 30°C on LB agar plates containing 100 µg/ml ampicillin, 10 mM potassium phosphate pH 7,0 and 0,4% glucose was resuspended in 10 µl 1 x PCR buffer (Super Taq™ DNA polymerase) heated to 99°C for 5 minutes, spun down 20 000 x g for 2 min.

35 5 µl of this supernatant was used as template in a PCR reaction (20 cycles) using the primers 5965 and 5966 and Super Taq™ DNA polymerase following the manufacturers instructions.

A PCR fragment corresponding to the expected size of *dsbA* was recovered from an agarose gel and digested with the restriction enzymes *EcoRI* and *PstI*.

5 The plasmid pPL1759, fig. 2, was digested with the restriction enzymes *PstI*-*EcoRI* and the large vector fragment was ligated to the PCR fragment. Ligation mixture was transformed into *Bacillus subtilis* DN1885. Selection for transformants and reisolation of those was performed on LB medium containing 10  
10  $\mu$ g Kanamycin/ml, 10 mM potassium phosphate pH 7.0, and 0.4% glucose.

DNA analysis of the plasmids from those cells using a DNA sequencing Kit (Sequenase™) showed the expected sequence of the  
15 promotor- and signal peptide encoding regions of *amyL* (P.L. Joergensen et al., *Gene*, 96, p. 37-41, 1990) fused to the above mentioned putative mature *dsbA* encoding region. This plasmid was termed pJA146 and a *B. subtilis* DN1885 strain harboring this plasmid was termed JA146. A plasmid map of pJA146 is shown in  
20 fig. 3.

#### Expression of *dsbA* in *Bacillus*

Strain JA146 was grown for 18 hours in Terrific Yeast medium at 37°C with 10  $\mu$ g/ml Kanamycin and 0.4% glucose in 20 ml M-tubes.  
25 at 280 rpm. Cells were harvested at 15 000 x g for 10 minutes and the supernatant was analysed for DsbA protein. SDS-PAGE of the supernatant showed that a protein of the size of mature DsbA protein was secreted into the media. Using the trypsin inhibitor assay it was shown that the DsbA protein has  
30 disulphide isomerase activity.

The N-terminal amino acid sequence was analysed as described above. The N-terminal amino acid of DsbA determined was : Ala-Ala-Gln-Tyr-Glu-Asp-Gly-Lys-Gln-

Example 3The effect of waving composition on hair

5       Testing of the P34H variant of Thioredoxin from *E. coli* for enzymatic waving of hair.

10       A tress of washed human Scandinavian hair (1 gram) was wetted with water and tightly wound on a curling roller. 1 ml of a solution with the following composition and a temperature of 30°C was applied to the tress:

4 mg/ml P34H Thioredoxin  
50 mM Phosphate buffer pH 7.5  
1 mM Reduced Gluthation (Sigma)

15       The tress was put in a plastic bag and incubated for 60 minutes at 30°C. Then the roller was removed and the hair was rinsed with water, dried with a cotton towel, combed and air dried.

20       Other tresses of hair was treated like above but without addition of the P34H Thioredoxin variant.

25       Treatment with the P34H variant of Thioredoxin gives a significant waving effect on the hair.

Example 4Waving effect on hair treated with DsbA

30       The following experiments compare the effect of waving on hair treated with DsbA from *E.coli* and bovine PDI.

35       Tresses of washed brown and fair human European hair (1 gram) was wetted with water and tightly wound on curling rollers. 1 ml of waving solution with the following composition and temperature of 30°C was applied to the tresses.

0.38 mg/ml DsbA

10mM Tris buffer pH 7.0 with 50mM NaCl

1 ml and 10 mM Reduced Gluthation (Sigma)

5 Tresses were put in plastic bags and stored for 60 minutes at 30°C. The hair was rinsed with water, dried with cotton towel, the rollers was removed, the hair was air dried and combed.

The results of the experiments are displayed in table A.

10 TABLE A

|       | DsbA mg/ml | $L_{after}/L_{before}$ |
|-------|------------|------------------------|
| Brown | 0          | 0.92                   |
|       | 0.38       | 0.73                   |
| Fair  | 0          | 0.93                   |
|       | 0.38       | 0.83                   |

15 The lenght of all tresses were measured before ( $L_{before}$ ) and after ( $L_{after}$ ) treatment.

$L_{after}/L_{before}$  is a measurement for the waving effect on the hair.

20 Example 5

Control of permanent waving

25 To control that the enzymatic waving was permanent and not only temporary the hair tresses were washed with a mild commercial shampoo.

30 After being treated, as described in example 4 and rinsed, the hair tresses were removed from the curling rollers and dried. Then control tresses and enzyme treated hair tresses were washed in either water or a mild shampoo, rinsed and air dried.

The results of the test are displayed in table B.

TABLE B

| DsbA<br>mg/ml | $L_{after}/L_{before}$<br>before shampoo-<br>ing | $L_{after}/L_{before}$<br>after shampoo-<br>ing |
|---------------|--------------------------------------------------|-------------------------------------------------|
| 0             | 0.92                                             | 0.92                                            |
| 0.38          | 0.73                                             | 0.73                                            |

5

$L_{after}/L_{before}$  is a measurement for the waving effect on the hair.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: NOVO NORDISK A/S
- (B) STREET: Novo Alle
- (C) CITY: Bagsvaerd
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2880
- (G) TELEPHONE: +45 4444 8888
- (H) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: A method of producing a protein  
disulfide  
redox agent

(iii) NUMBER OF SEQUENCES: 4

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30B

(EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCGTCGACTT ATTTTTTCTC GGACAGATAT T  
31

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear



30

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Primer"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCATCGATGA AAAAGATTTG GCTGGCGCT  
29

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 45 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Primer"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCTCATTATG CATCAGCGGC GCGCAGTAT GAAGATGGTA AACAG 45

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Primer"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCGAATTCGT CGACTTATTT TTTCTCGG  
28

Table 1:

|            |             |                                   |            |
|------------|-------------|-----------------------------------|------------|
|            | 1           |                                   | 50         |
| Pdi_Mouse  | .....MLS    | RALLCLALAW AARVGADALE EEDNVVLVKK  | SNFEEALAAH |
| Pdi_Rat    | .....MLS    | RALLCLALAW AARVGADALE EEDNVVLVKK  | SNFAEALAAH |
| Pdi_Bovin  | .....MLR    | RALLCLALTA LFRAGAGAPD EEDHVLVLHK  | GNFDEALAAH |
| Pdi_Human  | .....MLR    | RALLCLAVAA LVR..ADAPE EEDHVLVLRK  | SNFAEALAAH |
| Pdi_Rabbit | .....MLR    | RAVLCLALAV TA.GWAWAAE EEDNVVLVKS  | SNFAEALAAH |
| Pdi_Chick  | .....       | .....EPL EEDGVLVLRA               | ANFEQALAAH |
| Pdi_Yeast  | MKFSAGAVLS  | WSSLLLASSV FAQQEAVAPE DSA.VVKLAT  | DSFNEYIQSH |
|            | 51          |                                   | 100        |
| Pdi_Mouse  | KYLLVEFYAP  | WCGHCKALAP EYAKRAAKLK AEGSEIRLAK  | VDATEESDLA |
| Pdi_Rat    | NYLLVEFYAP  | WCGHCKALAP EYAKAAAKLK AEGSEIRLAK  | VDATEESDLA |
| Pdi_Bovin  | KYLLVEFYAP  | WCGHCKALAP EYAKAAGKLK AEGSEIRLAK  | VDATEESDLA |
| Pdi_Human  | KYLLVEFYAP  | WCGHCKALAP EYAKAAGKLK AEGSEIRLAK  | VDATEESDLA |
| Pdi_Rabbit | KHLLVEFYAP  | WCGHCKALAP EYAKAAGKLK AEGSDIRLAK  | VDATEESDLA |
| Pdi_Chick  | RHLLVEFYAP  | WCGHCKALAP EYAKAAQLK AEGSEIRLAK   | VDATEEELA  |
| Pdi_Yeast  | DLVLAEFFAP  | WCGHCKNMAP EYVKAETL. .VEKNITLAQ   | IDCTENQDLC |
|            | 101         |                                   | 150        |
| Pdi_Mouse  | QQYGVRGYPT  | IKFFKNGDTA SPKEYTAGRE ADDIVNWLKK  | RTGPAATTLS |
| Pdi_Rat    | QQYGVRGYPT  | IKFFKNGDTA SPKEYTAGRE ADDIVNWLKK  | RTGPAATTLS |
| Pdi_Bovin  | QQYGVRGYPT  | IKFFKNGDTA SPKEYTAGRE ADDIVNWLKK  | RTGPAASTLS |
| Pdi_Human  | QQYGVRGYPT  | IKFFRNGDTA SPKEYTAGRE ADDIVNWLKK  | RTGPAATTLR |
| Pdi_Rabbit | QQYGVRGYPT  | IKFFKNGDTA SPKEYTAGRE ADDIVNWLKK  | RTGPAATTLA |
| Pdi_Chick  | QQFGVRGYPT  | IKFFRNGDKA APREYTAGRE ADDIVSWLKK  | RTGPAATTLT |
| Pdi_Yeast  | MEHNIPGFPS  | LKIFKNSDVN NSIDYEGPRT AEAIVQFMK   | QSQPAVAVVA |
|            | 151         |                                   | 200        |
| Pdi_Mouse  | DTAAAESLVD  | SSEVTVIGFF KDVESDSAKQ FLLAAEAIDD  | IPFGITSNSG |
| Pdi_Rat    | DTAAAESLVD  | SSEVTVIGFF KDAGSDSAKQ FLLAAEAVDD  | IPFGITSNSD |
| Pdi_Bovin  | DGAAAEALVE  | SSEVAVIGFF KDMESDSAKQ FFLAAEVIDD  | IPFGITSNSD |
| Pdi_Human  | DGAAAESLVE  | SSEVAVIGFF KDVESDSAKQ FLQAAEAIDD  | IPFGITSNSD |
| Pdi_Rabbit | DSAAAESLVE  | SSEVAVIGFF KDVESDAAKQ FLLAAEATDD  | IPFGLTASSD |
| Pdi_Chick  | DAAAAE TLVD | SSEVVVIGFF KDVTSDAAKE FLLAAESVDD  | IPFGISSAD  |
| Pdi_Yeast  | DLPAYLANET  | FVTPVIVQSG KIDADFNATF YSMANKHFND  | YDFVSAENAD |
|            | 201         |                                   | 250        |
| Pdi_Mouse  | VFSKYQLDKD  | GVVLFKKFDE GR..NNFEGE ITKEKLLD.F  | IKHNQLPLVI |
| Pdi_Rat    | VFSKYQLDKD  | GVVLFKKFDE GR..NNFEGE ITKEKLLD.F  | IKHNQLPLVI |
| Pdi_Bovin  | VFSKYQLDKD  | GVVLFKKFDE GR..NNFEGE VTKEKLLD.F  | IKHNQLPLVI |
| Pdi_Human  | VFSKYQLDKD  | GVVLFKKFDE GR..NNFEGE VTKENLLD.F  | IKHNQLPLVI |
| Pdi_Rabbit | VFSRYQVHQD  | GVVLFKKFDE GR..NNFEGE VTKEKLLD.F  | IKHNQLPLVI |
| Pdi_Chick  | VFSKYQLSQD  | GVVLFKKFDE GR..NNFEGD LTKDNLLN.F  | IKSNQLPLVI |
| Pdi_Yeast  | ..DDFKL...  | SIYLP SAMDE PVVYNGKKAD IADADVFEKW | LQVEALPYFG |
|            | 251         |                                   | 300        |
| Pdi_Mouse  | EFTEQTAPKI  | FGGEIKTHIL LFLPKSVSDY DGKLSSFKRA  | AEGF..KGKI |
| Pdi_Rat    | EFTEQTAPKI  | FGGEIKTHIL LFLPKSVSDY DGKLSNFKKA  | AEGF..KGKI |
| Pdi_Bovin  | EFTEQTAPKI  | FGGEIKTHIL LFLPKSVSDY EGKLSNFKKA  | AESF..KGKI |
| Pdi_Human  | EFTEQTAPKI  | FGGEIKTHIL LFLPKSVSDY DGKLSNFKTA  | AESF..KGKI |
| Pdi_Rabbit | EFTEQTAPKI  | FGGEIKTHIL LFLPRSAADH DGKLSGFKQA  | AEGF..KGKI |
| Pdi_Chick  | EFTEQTAPKI  | FGGEIKTHIL LFLPKSVSDY EGKLDNFKTA  | AGNF..KGKI |
| Pdi_Yeast  | EIDGSVFAQY  | VESGLPLGYL FY.....ND EEELEEYKPL   | FTELAKKNRG |

table 1 (continued)

|           |                                                       |             |     |
|-----------|-------------------------------------------------------|-------------|-----|
|           | 301                                                   |             | 350 |
| Pdi_Mouse | LFIFIDSDHT DNQRILEFFG LKKEECPAVR LITLEEEM..           | .....TKY    |     |
| Pdi_Rat   | LFIFIDSDHT DNQRILEFFG LKKEECPAVR LITLEEEM..           | .....TKY    |     |
| Pdi_Bovin | LFIFIDSDHT DNQRILEFFG LKKEECPAVR LITLEEEM..           | .....TKY    |     |
| Pdi_Human | LFIFIDSDHT DNQRILEFFG LKKEECPAVR LITLEEEM..           | .....TKY    |     |
| Pdi_Rabit | LFIFIDSDHA DNQRILEFFG LKKEECPAVR LITLEEEM..           | .....TKY    |     |
| Pdi_Chick | LFIFIDSDHS DNQRILEFFG LKKEECPAVR LITLEEEM..           | .....TKY    |     |
| Pdi_Yeast | LMNFVSIDAR KFGRHAGNLN M.KEQFPLFA IHDMTEDLKY GLPQLSEAF |             |     |
|           | 351                                                   |             | 400 |
| Pdi_Mouse | KPESELTAE K..ITEFCHR FLEGKIKPHL MSQVPEDWD             | KQPVKVLVGA  |     |
| Pdi_Rat   | KPESELTAE K..ITQFCHH FLEGKIKPHL MSQELPEDWD            | KQPVKVLVVGK |     |
| Pdi_Bovin | KPESELTAE K..ITEFCHR FLEGKIKPHL MSQELPDDWD            | KQPVKVLVVGK |     |
| Pdi_Human | KPESELTAE R..ITEFCHR FLEGKIKPHL MSQERAGDWD            | KQPVKVPVVGK |     |
| Pdi_Rabit | KPESELTAE G..ITEFCQR FLEGKIKPHL MSQELPEDWD            | RQPVKVLVVGK |     |
| Pdi_Chick | KPEDDLAD K..IKEFCNK FLEGKIKPHL MSQDLPEDWD             | KQPVKVLVVGK |     |
| Pdi_Yeast | DELSKIVLE SKAIESLVKD FLKGDASPIV KSQEIFENQD            | S.SVFQLVVGK |     |
|           | 401                                                   |             | 450 |
| Pdi_Mouse | NFEEVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.           | KDHENIIIAK  |     |
| Pdi_Rat   | NFEEVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.           | KDHENIVIAK  |     |
| Pdi_Bovin | NFEEVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.           | KDHENIVIAK  |     |
| Pdi_Human | NFEDVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.           | KDHENIVIAK  |     |
| Pdi_Rabit | NFEEVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.           | KEHQDIVIAK  |     |
| Pdi_Chick | NFEEVAFDEN KNVFVEFYAP WCGHCKQLAP IWDKLGETY.           | RDHENIVIAK  |     |
| Pdi_Yeast | NHDEIVNDPK KDVLVLYYAP WCGHCKRLAP TYQELADTYA           | NATSDVLIAC  |     |
|           | 451                                                   |             | 500 |
| Pdi_Mouse | MDSTANEVEA VKVHSFPTLK FFPASADRTV IDYNGERTLD           | GFKKFLES GG |     |
| Pdi_Rat   | MDSTANEVEA VKVHSFPTLK FFPASADRTV IDYNGERTLD           | GFKKFLES GG |     |
| Pdi_Bovin | MDSTANEVEA VKVHSFPTLK FFPASADRTV IDYNGERTLD           | GFKKFLES GG |     |
| Pdi_Human | MDSTANEVEA VKVHSFPTLK FFPASADRTV IDYNGERTLD           | GFKKFLES GG |     |
| Pdi_Rabit | MDSTANEVEA VKVHSFPTLK FFPAGPGRTV IDYNGERTLD           | GFKKFLES GG |     |
| Pdi_Chick | MDSTANEVEA VKIHSFPTLK FFPAGSGRNV IDYNGERTLE           | GFKKFLES GG |     |
| Pdi_Yeast | LDHTENDVRG VVIEGYPTIV LYPGGKKSES VVYQGSRLD            | SLPDLFIKENG |     |
|           | 501                                                   |             | 538 |
| Pdi_Mouse | QDGAGDDEDL .DLEE..ALE PDME..DDD QKAVKDEL              |             |     |
| Pdi_Rat   | QDGAGDNDL .DLEE..ALE PDME..DDD QKAVKDEL               |             |     |
| Pdi_Bovin | QDGAGDDDL EDLEE..AEE PDLEE..DDD QKAVKDEL              |             |     |
| Pdi_Human | QDGAGDDDL EDLEE..AEE PDME..DDD QKAVKDEL               |             |     |
| Pdi_Rabit | QDGAGDEDL EDLEE..AEE PDLEE..DDD QKAVRDEL              |             |     |
| Pdi_Chick | QDGAAADDL EDLET..DEE TDL EEGDDDE QKI QKDEL            |             |     |
| Pdi_Yeast | HFDVDGKALY EEAQEKAEE ADADAELADE EDALHDEL              |             |     |

Table 2:

|         |             |            |               |                 |            |
|---------|-------------|------------|---------------|-----------------|------------|
| Alfalfa | M-AKNVAIFG  | LLFSLLLLVP | SQIFA-----    | -----EES        | STDAKE---- |
| Oryzae  | MRTFAPWIL-  | --SLLGASA- | --VAS-----    | -----AADA       | TAEAPS---- |
| Yeast   | MKFSAGAVLS  | WSSLLLASS- | --VFA-----    | -----QQA        | VAPEDS---- |
| Bovine  | M-LRRA-LLC  | --LALTALF- | --RAG-----    | -----AGA        | PDEEDH---- |
| Rat     | M-LSRA-LLC  | --LALAWAA- | --RVG-----    | -----ADA        | LEEEDN---- |
| Mouse   | MKLRKAWLLV  | LLLALTQLLA | AASAGDAQED    | TSDTENATEE      | EEEEDDDDLE |
|         |             |            | -----FVL----- |                 |            |
|         |             |            | -----DVV----- |                 |            |
|         |             |            | -----AVV----- |                 |            |
|         |             |            | -----VL-----  |                 |            |
|         |             |            | -----VL-----  |                 |            |
|         | VKEENGWVWL  | NDGNFDNFVA | DKDTVLLEFY    | APWCGHCKQF      | APEYEKIAST |
|         |             |            |               | -----TLDNT----- |            |
|         |             |            |               | -----SLTGD----- |            |
|         |             |            |               | -----KLATD----- |            |
|         |             |            |               | -----VLHKG----- |            |
|         |             |            |               | -----VLKKS----- |            |
|         | LKDNDPPIAV  | AKIDATSASM | LASKFDVSGY    | PTIKILKKGQ      | AVDYDGSRTQ |
|         |             |            | -----NF       | HDTVKKHDFI      | VVEFYAPWCG |
|         |             |            | -----TF       | ETFVKEHDLV      | LAEFFAPWCG |
|         |             |            | -----SF       | NEYIQSHDLV      | LAEFFAPWCG |
|         |             |            | -----NF       | DEALAAHKYL      | LVEFYAPWCG |
|         |             |            | -----NF       | AEPAAHNYLL      | VEFY-APWCG |
|         | EEIVAKVREV  | SQPDWTPPPE | VTLSTKDNF     | DDVNNADII       | LVEFYAPWCG |
|         | HCKKLAPEYE  | KAASILSTHE | PPVVLAKVDA    | NEEHNKDLAS      | ENDVKGFPTI |
|         | HCKALAPKYE  | QAATELKEKN | IPL--VKVDC    | TEEEA--LCR      | DQGVGYPTL  |
|         | HCKNMAPEYV  | KAAETLVEKN | ITL--AQIDC    | TENQD--LCM      | EHNIPGFPSL |
|         | HCKALAPEYA  | KAAGKLKAE  | SEIRLAKVDA    | TEESD--LAQ      | QYGVRGYPTI |
|         | HCKALAPEYA  | KAAAKLKAE  | SEIRLAKVDA    | TEESD--LAQ      | QYGVRGYPTI |
|         | HCKKLAPEYE  | KAAKELSKRS | PPIPLAKVDA    | TEQTD--LAK      | RFDVSGYPTL |
|         | KIFRNGG-KN  | IQEYKGPREA | EGIVEYLKKQ    | SGPAS-TEIK      | SADDATAFVG |
|         | KIFRGLDAVK  | P--YQGARQT | EAIVSYMKQ     | SLPAV-SPVT      | PENLEE-IKT |
|         | KIFKNRDVNN  | SIDYEGPRTA | EAIVQFMKKQ    | SQPAV-AVVA      | DLPAYL-ANE |
|         | KFFKNGDTAS  | PKEYTAGREA | DDIVNWLKKR    | TGPAA-STLS      | DGAAAEALVE |
|         | KFFKNGDTAS  | PKEYTAGREA | DDIVNWLKKR    | TGPAA-TTLS      | DTAAAESLVD |
|         | KIFRKG---R  | PFDYNGPREK | YGIVDYMIEQ    | SGPPSKEILT      | LKQVQEFLKD |
|         | DNKVVIIVGVF | PKFSGEEYDN | FIALAEKLRS    | DYDFAHTLNA      | KHLPGKDSSV |
|         | MDKIVVIGYI  | ASDDQTANDI | FTTFAESQRD    | NYLFAATSDA      | SI--AKAEGV |
|         | TFVTPVIVQS  | GKIDADFNAT | FYSMANKHFN    | DYDFVSAENA      | DD--DFKLSI |
|         | SSEVAVIGFF  | KMESDSAKQ  | FLLAAEVI-D    | DIPFGITSNS      | DV--FSKYQL |
|         | SSEVTVIGFF  | KDAGSDSAKQ | FLLAAEAV-D    | DIPFGITSNS      | DV--FSKYQL |
|         | GDDVVIIGLF  | QGDGDPAYLQ | YQDAANNLRE    | DYKFHHTFSP      | EIAKFLKVS  |

table 2 (continued)

SGPVVRLFKP FDELFVDS-- -KDFNVEALE KFIEESSTPI VTVFNNEPSN  
 KQPSIVLYKD FDEKKATYDG EIEQDALLSW VKTASTPLVG ELGPETYSGY  
 YLPSAM--DE PVVYNGKKAD IADADVFEKW LQVEALPYFG EIDGSVFAQY  
 DKDGVVLFKK FD---EGR-- -NNFEGEVTK EKLLDFIKHN QLPLVIEFTE  
 DKDGVVLFKK FD---EGR-- -NNFEGEITK EKLLDFIKHN QLPLVIEFTE  
 GKLVLTPEK FQSKYEPRFH VMDVQGSTE AIAKDYVVKH ALPLVGHRTK  
  
 HPFVVKFENS PNAKAMLFIN FTTEGAESFK TKYHEVAEQY KQQGV-SFLV  
 ITAGIPLAYI FAETKEEREQ FTEEFKFAIE KHKGSINIIVT IDAKLYGAHA  
 VESGLPLGYL FYNDEEELEE YKPLFTELAK KNRGLMNFVS IDARKFGRHA  
 QTAPKIFGGE IKTHILLFLP KSVSDYEGKL SNFKKAAESF KGKILFIFID  
 QTAPKIFGGE IKTHILLFLP KSVSDYDGKL SNFKKAAEGF KGKILFIFID  
 SNAKRYSKR PLVVVYYSVD FSFDYRAATQ FWRNKVLEVA KDFPEYTFAI  
  
 GDVESSQAF QYFGLKEEQV PLI--IIQHN DGKKFFKPN- --LELDQLPT  
 GNLNLDPSKF PAFAIQDPEK NAKY----- --PYDQSKE- --VKAKDIGK  
 GNLNMK-EQF PLFAIHDMTE DLKYGLPQLS EEAFDELSDK IVLESKAIES  
 SDHTDNQRIL EFFGLKKEEC PAVR-LITLE EEMTKYPES DELTAEKITE  
 SDHTDNQRIL EFFGLKKEEC PAVR-LITLE EEMTKYPES DELTAEKITQ  
 ADEEDYATEV KDLGL-SESG EDVN-AAILD ESGKKFAMEP EEFDSDTLRE  
  
 WLKAYKDGKV EPPVKSEPIP ETNN-EPVKV VVGQTLLEDV FKSGKNVLIE  
 FIQDVLDDKV EPSIKSEAI ETQE-GPVTV VVAHSYKDLV LDNEKDVLLE  
 LVKDFLKGDA SPIVKSQEIF ENQD-SSVFQ LVGKNHDEIV NDPKKDVLVL  
 FCHRFLEGKI KPHLMSQELP DDWDKQPVKV LVGKNFEEVA FDEKKNVFVE  
 FCHHFLEGKI KPHLMSQELP EDWDKQPVKV LVGKNFEEVA FDEKKNVFVE  
 FVTAFKKGKL KPVIKSQPVP KN-NKGPVKV VVGKTFDAIV MDPKKDVLIE  
  
 FYAPWCGHCK QLAPILDEVA VSFQS-DADV VIAKLDTAN DIPTDTFDVQ  
 FYAPWCGHCK ALAPKYEELA SLYKD-IPEV TIAKIDATAN DV--PD-SIT  
 YYAPWCGHCK RLAPTYQELA DTYANATSDV LIAKLDHTEN DV--RGVVIE  
 FYAPWCGHCK QLAPIWDKLG ETYKD-HENI VIAKMDSTAN EV--EAVKVH  
 FYAPWCGHCK QLAPIWDKLG ETYKD-HENI VIAKMDSTAN EV--EAVKVH  
 FYAPWCGHCK QLEPIYTSLG KKYKG-QKDL VIAKMDATAN DITNDQYKVE  
  
 GYPTLYFRSA SGK--LSQYD GGRTKEDIIE FIE-----K NKDKTGAHQ  
 GFPTIKLFAA GAKDSPVEYE GSRTVEDLAN FVK-----E NGKHKVDAL  
 GYPTIVLYPG GKKSESVVYQ GSRSLDSLFD FIK-----E NGHFDVDGKA  
 SFPTLKFFPA SADRTVIDYN GERTLDGFKK FLESGGQDGA GDDDDLEDLE  
 SFPTLKFFPA SADRTVIDYN GERTLDGFKK FLESGRQDGA GDNDLDLEE  
 GFPTIYFAPS GDKKNPI--- -----K F-----E GGNRDLEHLS  
  
 EVEQPKAAQ PE----- AEQPKDEL  
 VDPKKEQESG DATETRAASD ETETPAATSD DKSEHDEL  
 LYEEAQEKAA EEAEDAEE ADADAELADE EDAIHDEL  
 EAEFPDLEED DD----- QKAVKDEL  
 ALE-PDMEED DD----- QKAVKDEL  
 KF--ID-EHA TK----- RSRTKEEL

PATENT CLAIMS

1. A protein disulfide redox agent comprising a sequence A  
R<sup>1</sup>-Cys-X-Y-Cys-R<sup>2</sup> (A)

wherein R<sup>1</sup> and R<sup>2</sup> each independently are different or the same  
5 amino acid sequences, preferably such sequences as are known  
from naturally occurring protein disulfide redox agent, X and Y  
each independently are any naturally occurring amino acid  
residues; with the exception of agents, wherein R<sup>1</sup> and R<sup>2</sup> are  
identical to known naturally occurring protein disulfide  
10 isomerases, protein disulfide oxidoreductase or thioredoxins  
and X and Y corresponds to the naturally occurring amino acid  
residues, and the variant of TRX from *E. coli*, wherein His has  
been substituted for Pro as Y (*E. coli* P34H).

2. The protein disulfide redox agent of claim 1, wherein R<sup>1</sup>  
15 and R<sup>2</sup> each independently are different or the same amino acid  
sequences having lengths between 5 and 500 amino acid residues,  
preferably between 25 and 250 amino acid residues, or between  
40 and 150 amino acid residues.

3. The protein disulfide redox agent of claim 1 or 2, wherein  
20 X is chosen among Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly,  
Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg and His, and  
Y is chosen among Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly,  
Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg and His.

4. The protein disulfide redox agent of claim 1 or 3, wherein  
25 X is chosen among Glu, Ala, Ser, Asp, Pro or Val and Y is  
chosen among Arg, Lys, Asn, Gln and Tyr.

5. The protein disulfide redox agent of claim 1 or 3, wherein  
X is Gly, Val or Ala, while Y is any of the 20 other naturally  
occurring amino acid.

6. The protein disulfide redox agent of claim 5, wherein Y is Arg, Lys, Asn, Gln, His or Tyr.
7. The protein disulfide redox agent of claim 1 or 3, wherein Y is either His, Pro or Tyr, while X is any of the 20 other naturally occurring amino acids.
8. The protein disulfide redox agent of claim 7, wherein X is Glu, Ala, Ser, His or Asp.
9. The protein disulfide redox agent of any of the claims 1 to 8, wherein said agent is a variant of a protein disulfide isomerase, protein disulfide oxidoreductase or thioredoxin.
10. The protein disulfide redox agent of claim 9, wherein not all sequences (A) in a specific variant or mutein have been changed, or have not been changed in the same way.
11. The protein disulfide redox agent of claim 9, wherein all sequences (A) in a specific variant or mutein have been changed in the same way.
12. The protein disulfide redox agent of claims 9, 10 or 11, wherein the parent molecule has been divided or truncated so as to provide for a smaller number of sequences (A) in said agent.
13. The protein disulfide redox agent of claim 12, comprising a PDI subunit with 2 (A) sequences.
14. The protein disulfide redox agent of claim 12, comprising a split PDI, dsbA or a TRX unit with only one (A) sequence.
15. The protein disulfide redox agent of any of the claims 9 to 14, wherein at least one of  $R^1$  and  $R^2$  are different from those of the parent molecule.

16. The protein disulfide redox agent of claim 15, wherein at least one of  $R^1$  and  $R^2$  are shorter or longer than their parent entities.
17. The protein disulfide redox agent of claim 16, wherein  $R^1$  and/or  $R^2$  is(are) characterized by lacking or having inserted therein amino acids at either the N-terminus, C-terminus, internally, or any combination hereof, as compared to naturally occurring protein disulfide redox agents.
18. The protein disulfide redox agent of any of the preceding 10 claims 9 to 17, having a redox potential differing by at least 1 mV as compared to the redox potential of the original protein.
19. A DNA construct encoding a protein disulfide redox agent according to any of the claims 1 to 18.
- 15 20. The DNA construct according to claim 19, wherein the DNA sequence is degenerate as a result of the genetic code.
21. A recombinant expression vector comprising a DNA construct according to any of claims 19 or 20.
22. The vector of claim 21, wherein said DNA construct is 20 operably linked to a promoter sequence and optionally to a sequence encoding a secretion signal.
23. A cell comprising a DNA construct according to any of claims 19 or 20 or a vector according to any of claims 21 or 22.
- 25 24. A cell according to claim 23, which is a microbial cell.
25. The cell according to claim 24, which is a bacterial cell or a fungal cell.



26. The cell according to claim 25, in which the bacterial cell is a cell of a gram-positive bacterium, e.g. of the genus *Bacillus* or *Streptomyces* or a cell of a gram-negative bacterium, e.g. of the genus *Escherichia*, and the fungal cell is a yeast cell, e.g. of the genus *Saccharomyces*, or a cell of a filamentous fungus, e.g. of the genus *Aspergillus* or *Fusarium*.

27. The cell according to claim 26, wherein said *Escherichia* is *E. coli*.

28. The cell according to claim 26, wherein said *Aspergillus* is *Aspergillus niger*, *Aspergillus oryzae*, or *Aspergillus nidulans*.

29. The cell according to claim 26, wherein said *Bacillus* is *Bacillus licheniformis*, *Bacillus lentus*, or *Bacillus subtilis*.

30. A method of producing a recombinant protein disulfide redox agent as defined in any of the claims 1 to 18, wherein a cell according to any of the claims 23 to 29, is cultured under conditions conducive to the production of the protein disulfide redox agent and the protein disulfide redox agent is subsequently recovered from the culture.

20

31. The method according to claim 30, in which the recombinant protein disulfide redox agent is expressed in the form of a proenzyme and the cell is cultured in the presence of a proteolytic enzyme capable of converting the proenzyme of the protein disulfide redox agent into a mature enzyme.

32. A composition of matter comprising (i) a protein disulfide redox agent, optionally (ii) at least one redox partner, and optionally (iii) one or more other enzymes.

33. The composition of claim 32, wherein said protein disulfide redox agent (i) is a naturally occurring protein disulfide isomerase, -oxidase, -reductase, -oxidoreductase, a thioredoxin, a sulfhydryl oxidase, -oxidoreductase, -reductase, or -

transferase, capable of catalyzing the reduction/oxidation of protein disulfide linkages.

34. The composition of claim 32, wherein said original protein is a thioredoxin naturally produced by *Escherichia coli* and 5 proline at position 34 has been substituted with histidine (*E. coli* P34H).

35. The composition of claim 32, wherein said original protein is DsbA naturally produced by *Escherichia coli*.

10 36. The composition of claim 32, wherein said protein disulfide redox agent (i) is a derivative defined as in any of the claims 1 to 17.

37. The composition of any of the claims 32 to 36, wherein said redox partner (ii) is an organic or inorganic reductant.

15 38. The composition of claim 37, wherein said organic reductant is selected from the group comprising glutathione, L-cysteine, dithiothreitol, 2-mercaptoethanol, thioglycolic acid, L-cysteine ethylester,  $\beta$ -mercaptoethylamine, mercaptosuccinic acid,  $\beta$ -mercaptopropionic acid, dimercapto adipic acid, 20 thiomalic acid, thioglycolamides, glycol thioglycolate, glycerol thioglycolate, thiolactic acid and salts thereof.

39. The composition of claim 37, wherein said inorganic reductant is selected from the group comprising sulfite and bisulfite.

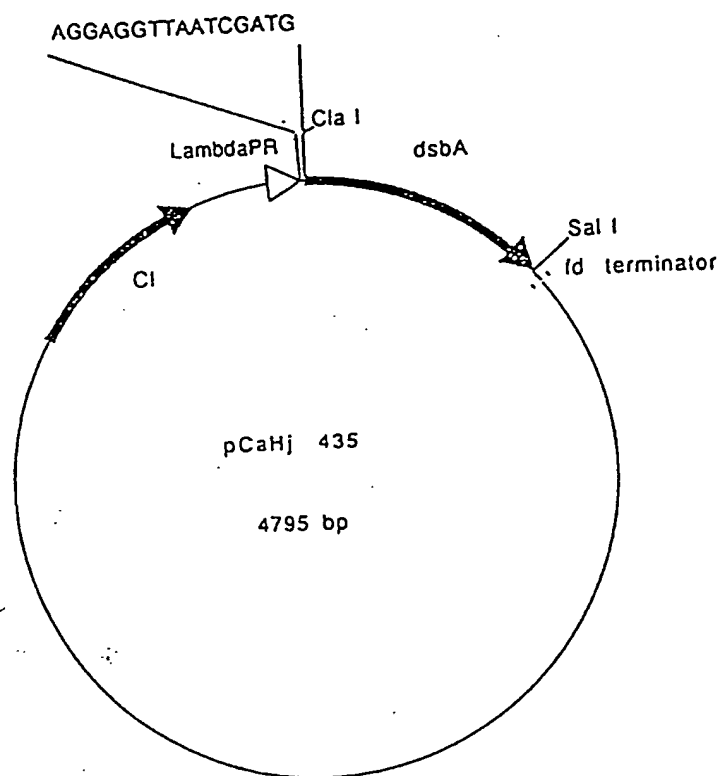
25 40. The composition of any of the preceding claims, in which said other enzyme (iii) is a protease, a lipase, an amylase, a transglutaminase, or another protein disulfide redox agent.

41. A process for treating scleroproteins which comprises applying the composition of claim 32 to 40 to the 30 scleroprotein.

42. The process of claim 41, wherein said scleroprotein is human hair or animal hair.
43. The process of claim 42, wherein said process involves waving, straightening, degrading or softening of said hair.
- 5 44. A process for the cleaning of fabrics involving the application of the composition of claim 32 to 40 to said fabrics.
45. The process of claim 44 also involving treatment with a detergent.
- 10 46. A process for thickening and/or gelation of food involving application of the composition of claim 32 to 40 to the food.
47. A process for the dissolution of lung gels involving application of the composition of claim 32 to 40 to the lungs.
- 15 48. A process for the alleviation of certain eye conditions involving application of the composition of claim 32 to 40 to the eyes.

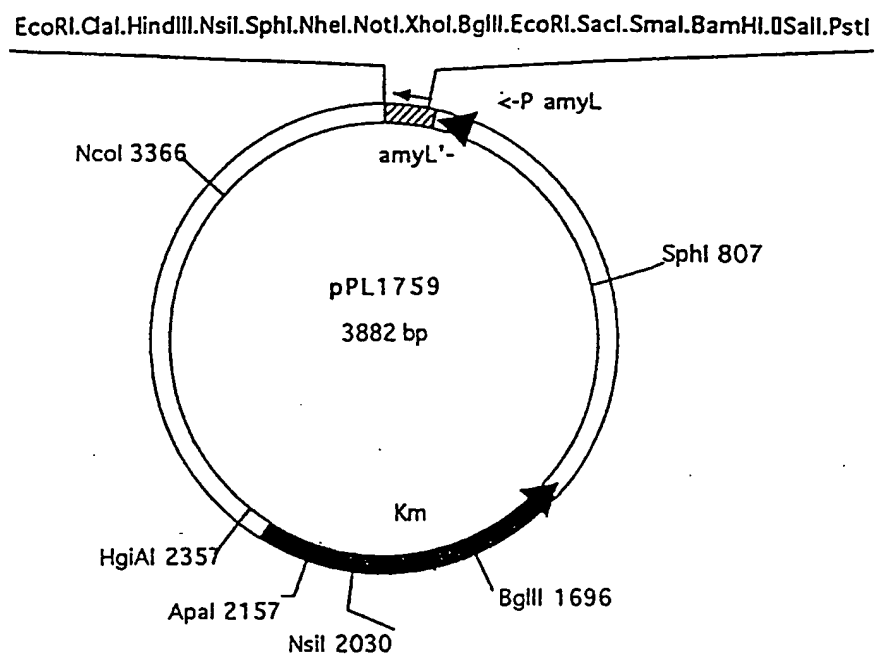
1/3

Figure 1:



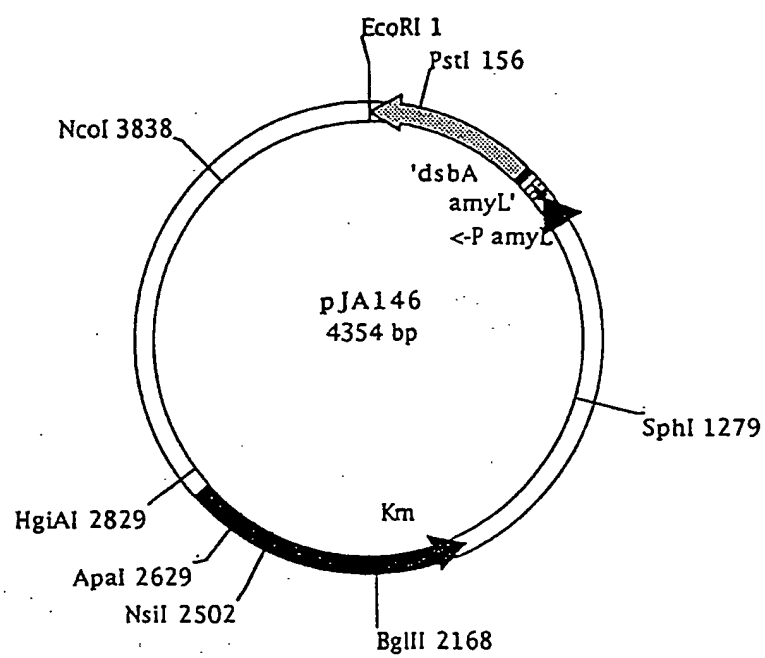
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Figure 2:



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Figure 3:



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00265

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/00, C12N 9/02, C12N 9/90, A61K 7/06, A61K 7/09  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, WPIL, WPI, EMBASE, US PATENTS FULLTEXT DATABASES

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                | Relevant to claim No. |
|-----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | Dialog Information Services, file 155, Medline, Dialog Accession no.08554420, Nikkola M. et al: "Crystal structure analysis of a mutant Escherichia coli thioredoxin in which lysine 36 is replaced by glutamic acid", Biochemistry (UNITED STATES) May 18 1993, 32 (19) p5093-8. | 1-31,36-40            |
| Y         | --                                                                                                                                                                                                                                                                                | 41-43                 |

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

25 November 1994

Date of mailing of the international search report

05 -12- 1994

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00265

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                              |                       |
|-------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                             | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                           | Relevant to claim No. |
| X                                                     | Dialog Information Services, file 155, Medline,<br>Dialog Accession no.07635193, Krause G. et al:<br>"Substitution of the conserved tryptophan 31 in<br>Escherichia coli thioredoxin by site-directed<br>mutagenesis and structure-function analysis",<br>J. Biol. Chem. (UNITED STATES) Mar 5 1991, 266(7)<br>p4056-66.                                     | 1-31,36-40            |
| Y                                                     | --                                                                                                                                                                                                                                                                                                                                                           | 41-43                 |
| X                                                     | J. Basic Microbiol., Volume 33, No 3, 1993,<br>Peter Minarik, "Influence of the different amino<br>acid substitutions in Escherichia coli thioredoxin<br>on the growth of bacteriophages T7 and f1",<br>page 213 - page 215, The exact date of publication<br>has not been elucidated                                                                        | 1-31,36-40            |
| Y                                                     | --                                                                                                                                                                                                                                                                                                                                                           | 41-43                 |
| X                                                     | Dialog Information Services, file 155, Medline,<br>Dialog Accession no. 07246967, Joelson T. et al:<br>"Modifications of the active center of T4<br>thioredoxin by site-directed mutagenesis",<br>J Biol. Chem (UNITES STATES) Feb 25 1990, 265 (6)<br>p3183-8                                                                                               | 1-31,36-40            |
| Y                                                     | --                                                                                                                                                                                                                                                                                                                                                           | 41-43                 |
| X                                                     | Dialog Information Services, file 155, Medline,<br>Dialog accession no. 08112497, Lundström J et al:<br>"A Pro to His mutation inactive site of thioredoxin<br>increases its disulfide isomerase activity 10 fold.<br>New refolding systems for reduced or randomly<br>oxidized ribonuclease", J Biol Chem (UNITED STATES)<br>MAY 5 (1992, 267 (13) P9047-52 | 32-35,37-40           |
| Y                                                     | --                                                                                                                                                                                                                                                                                                                                                           | 41-43                 |



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00265

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------|-----------------------|
| X         | EP, A1, 0272781 (THE UNIVERSITY OF READING),<br>29 June 1988 (29.06.88), claim 1   | 32-35, 37-43          |
| Y         | --                                                                                 | 41-43                 |
| X         | DE, A, 2141763 (HENKEL & CIE GMBH), 1 March 1973<br>(01.03.73), claim 1            | 41-43                 |
| Y         | --                                                                                 | 41-43                 |
| X         | DE, A, 2141764 (HENKEL & CIE GMBH), 1 March 1973<br>(01.03.73), claim 1            | 41-43                 |
| Y         | --<br>-----                                                                        | 41-43                 |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00265

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 1  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
claim 1 is not considered to be clear and concise since it is not specified which the intended modifications are or for which purpose (cf. PCT article 6). Therefore, the search may have been incomplete.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Motivation of Lack of Unity a Priori.

As is stated in Annex B to Administrative Instructions under the PCT, in force July 1, 1992 (PCT GAZETTE 1992, June 25, pages 7062-9, see page 7063 and example 5) unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features" -i.e. features that define a contribution which each of the inventions makes over the prior art.

A search for this "special technical feature" mentioned in PCT Rule 13.2 among the independent claims did not reveal such a unifying, novel technical feature for all independent claims. Accordingly, the following inventions were found:

Invention 1, claims 1-31, 36, 37-48 (partially), pertains to a protein disulfide redox agent comprising the sequence R<sup>1</sup>-Cys-X-Y-Cys-R<sup>2</sup> wherein R<sup>1</sup> and R<sup>2</sup> each independently are different or the same amino acid sequences, preferably such sequences as are known from naturally occurring protein disulfide redox agent, X and Y each independently are any naturally occurring amino acid residues; with the exception of agents, wherein R<sup>1</sup> and R<sup>2</sup> are identical to known naturally occurring protein disulfide isomerases, protein disulfide oxidoreductase or thioredoxins and X and Y corresponds to the naturally occurring amino acid residues, and the variant of TRX from E. coli, wherein His has been substituted for Pro as Y (E. coli P34H).

Invention 2, claims 32-35, 37-48 (partially) concerns a composition comprising a protein disulfide redox agent.

Motivation of Lack of Unity a Posteriori.

According to PCT rule 13.2 an international application shall relate to one invention only or a group of inventions so linked as to form a "single general inventive concept". Such a general inventive concept must fulfil the criteria of novelty and inventive step.

Initially, all the subject matters mentioned in claims 1-36, 37-48 (partially), were included in the search. However, it soon became obvious that pertinent prior art exists making it necessary to reconsider the technical relationship between the different solutions revealed in the claims.

The prior art is, for invention 1, represented by Dialog Information Services file 155 Medline Dialog Accession numbers 08554420, 07246967, 07635193 and J. Basic Microbiol., Volume 33, No 3, 1993, 213-215. These documents concerns modified thioredoxins produced by site-directed mutagenesis.

For invention 2, the prior art is represented by Dialog Information Services, file 155, Medline, Dialog Accession No, 08112497 and EP, A1, 0272781.

Thus, the state of the art reveals that the amino acid sequence of protein disulfide redox agents have been changed by substitution of one or more of the naturally occurring amino acids and that compositions are known which comprise a protein disulfide redox agent.

A search for a new common concept of invention has failed and, thus, several of the claims represent independent subject matter forming lack of unity with each other.

ISA has arrived at a principle of division based on the prior art:

Invention A, claims 1-36, 37-40 (partially), pertains to a protein disulfide redox agent comprising the sequence  $R^1$ -Cys-X-Y-Cys- $R^2$  wherein  $R^1$  and  $R^2$  each independently are different or the same amino acid sequences, preferably such sequences as are known from naturally occurring protein disulfide redox agent, X and Y each independently are any naturally occurring amino acid residues; with the exception of agents, wherein  $R^1$  and  $R^2$  are identical to known naturally occurring protein disulfide isomerases, protein disulfide oxidoreductase or thioredoxins and X and Y corresponds to the naturally occurring amino acid residues, and the variant of TRX from E. coli, wherein His has been substituted for Pro as Y (E. coli P34H).

Invention 2, claims 32-35 and 37-40 (partially), concerns a composition comprising a protein disulfide redox agent.

Invention B, claims 41-43 (partially), pertains to a process for treating scleroproteins.

Invention C, claims 44-45 (partially), concerns a process for the cleaning of fabrics.

Invention D, claim 46 (partially), concerns a process for thickening and/or gelation of food.

Invention E, claim 47 (partially), concerns a process for the dissolution of lung gels.

Invention F, claim 48 (partially), concerns a process for the alleviation of certain eye conditions.

The international search covers inventions A=1, 2 and B.

Notice that the search for invention B covers both compositions comprising natural protein disulfide redox agents and compositions comprising protein disulfide redox agents according to invention A=1. The search for invention B has mainly been focused on hair treatment.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

29/10/94

International application No.  
PCT/DK 94/00265

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)                       | Publication<br>date  |
|-------------------------------------------|---------------------|--------------------------------------------------|----------------------|
| EP-A1- 0272781                            | 29/06/88            | SE-T3- 0272781<br>DE-A- 3777779<br>US-A- 4853215 | 30/04/92<br>01/08/89 |
| DE-A- 2141763                             | 01/03/73            | NONE                                             |                      |
| DE-A- 2141764                             | 01/03/73            | NONE                                             |                      |